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SUBMERGED CITRIC ACID FERMENTATION OF FERROCYANIDE-TREATED BEET MOLASSES: MORPHOLOGY OF PELLETS OF ASPERGILLUS NIGER[1]

D. S. CLARK

Abstract

A study of the internal structure of *Aspergillus niger* pellets, grown during submerged citric acid fermentation of ferrocyanide-treated beet molasses, was made using botanical microtechniques. Under optimum fermentation conditions, each pellet developed as a round mass of mycelium of uniform consistency during the first 24 hours of the fermentation (mash sparged with air); subsequently (mash sparged with oxygen), a dense crust of growth formed at the periphery of the pellet and autolysis of cells at the center began. At the end of fermentation (144 hours), the pellet consisted of a shell of mycelium occupying less than 30% of the pellet volume. Changes in fermentation conditions were reflected in the density of peripheral growth and in the rate and extent of autolysis.

Introduction

Under conditions used in submerged citric acid fermentation of ferrocyanide-treated beet molasses, *Aspergillus niger* develops as spherical units of mycelium generally referred to as pellets (1–5, 8, 9). The external characteristics of pellets of different ages and produced under a variety of fermentation conditions were studied and found related in many instances to citric acid yield (2, 8, 9). Such observations were helpful in assessing the cause of some fermentation problems. The internal morphology of pellets, however, was not examined in detail.

Methods

The fermentation procedures and equipment used for growing the pellets have been described in detail (1, 2, 5, 8, 9). Briefly, mash for inoculum preparation and fermentation was prepared from Chatham beet molasses[2] diluted to 12% sugar concentration with tap water. The mash was adjusted to pH 6.0, sterilized, and treated while hot with potassium ferrocyanide. The precipitate formed was not removed. The concentration of ferrocyanide in the cooled mash was measured and, unless otherwise stated, adjusted to 15 p.p.m. (2). The poststerilization pH was adjusted to 6.5. "Standard" pellet-type inoculum (9) was prepared by adding 10^8 spores of *A. niger* NRC A-1-233 to 1500 milliliters of mash in 6-liter flasks and incubating the suspensions at 29° C for 18–24 hours on a rotary shaker. Fermentations were carried out at 31° C in Pyrex tower fermenters (5, 8). The mash was inoculated to contain 2×10^5 pellets/liter and sparged with air for the first 24 hours of fermentation, and with air, oxygen, or mixtures of air and oxygen for the remaining time, using

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[2]Canada and Dominion Sugar Co., Chatham, Ontario.

a gas flow rate of 300 ml/minute/liter. When an increased total pressure in the fermenters (above 1 atm) was used, this pressure was maintained automatically by a pneumatic pressure controller (1).

Numerous 140-hour fermentations were conducted to study the effect of fermentation age, ferrocyanide content of the fermentation mash, and oxygen pressure in the gas supply, on the internal morphology of pellets. In these tests, samples of 25 or more pellets were collected at various times during fermentation, washed in distilled water, immersed in Nawashin-type (Craf) killing and preserving fluid [0.5% chromic acid, 3.5% acetic acid, and 1.5% formaldehyde in water (6, p. 18)], and stored at 10° C.

Botanical microtechniques were used to process the pellets for microscopic examination. At least five pellets were selected from each killed sample, transferred to a 20% aqueous ethanol solution, passed stepwise through the ethanol–butanol dehydration series described by Sass (6, p. 23), infiltrated with paraffin wax (Tissuemat, melting point 52° C), and cast into molds. The embedded pellets were sliced with a rotary microtome into sections 15 microns thick. Eight to ten sections from the center of the pellets were floated on a few drops of an aqueous solution of egg albumin (7) on a microscope slide, warmed to 50° C, and straightened. The slides were placed in a 35° C oven for 24 hours and the dried sections then dewaxed and stained with crystal violet (6, p. 75). After cover glasses were cemented on the preparations, slides from each sample were examined under the microscope and the section best representing the average appearance of the group was photographed.

Results

Figures 1–3 show the external appearance of the three general types of pellets produced, each of which was associated with certain fermentation conditions and related to citric acid yield. A round, hard, cream-colored pellet was formed (Fig. 1) when conditions were optimum for high yields of citric acid (2). These grew from a diameter of 0.2–0.5 mm at the beginning of fermentation to 1.2–2.5 mm after 5 days, remained well separated throughout this time, and produced little filamentous growth at their surfaces. Large (up to 6 mm) irregularly shaped clumps of pellets were formed (Fig. 2) when the ferrocyanide concentration in mash was too low (below 10 p.p.m.) for optimum citric acid yield (2). Although these clumps were smooth and hard like the pellets in Fig. 1, they generally produced citric acid at a slower rate, presumably because the active mold surface area was less. Soft filamentous pellets that varied extensively in size and shape (Fig. 3) were formed when the mash contained no ferrocyanide at the start of fermentation. These produced little citric acid.

The internal morphological changes that occurred with increase in fermentation time, in pellets grown under conditions optimum for citric acid production, are shown in Figs. 4–9. The small pellets used as inoculum (Fig. 4) grew rapidly but underwent no marked changes in appearance during the first 20 hours of fermentation when the mash was sparged with air (Fig. 5). After 6 hours on oxygen (Fig. 6), however, growth at the edge of the pellets had become dense and cells at the center had begun to autolyze. After 42 hours

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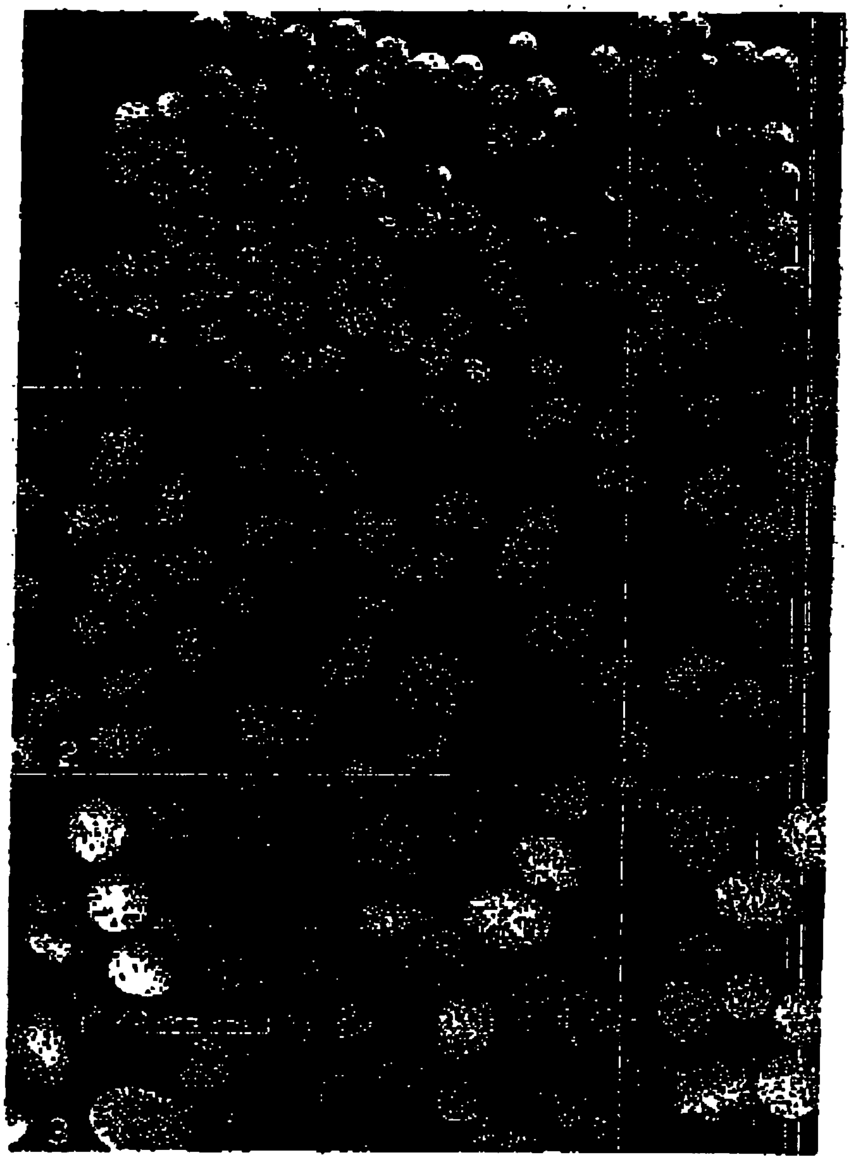

FIGS. 1–3. External appearance of types of pellets formed during submerged citric acid fermentation of beet molasses. Fermentation age 72 hours. Fig. 1. Hard, smooth pellets. Fig. 2. Clumped pellets. Fig. 3. Soft, filamentous pellets.

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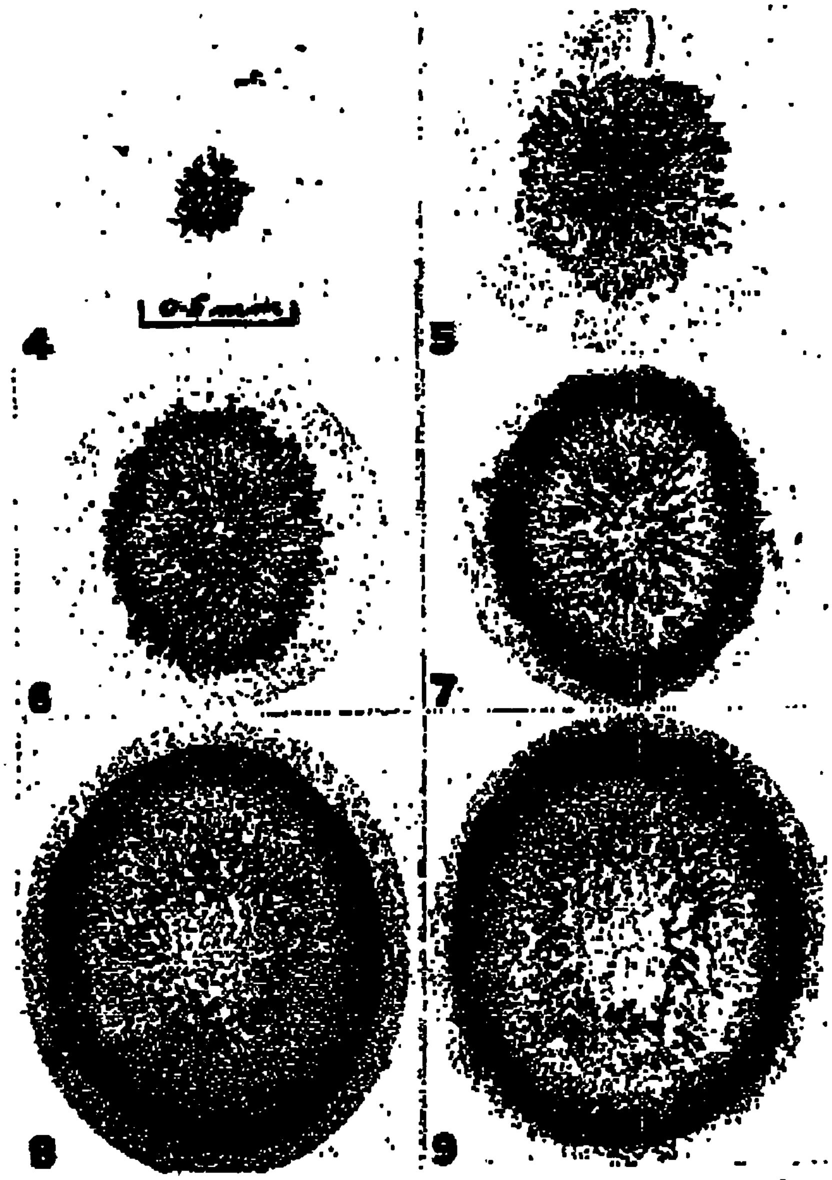
PLATE II



FIGS. 4–9. Photomicrographs of cross sections of pellets showing changes in morphology with fermentation age. Fermentation age: Fig. 4, 0 hour; Fig. 5, 20 hours; Fig. 6, 25 hours; Fig. 7, 42 hours; Fig. 8, 72 hours; Fig. 9, 100 hours.

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PLATE II

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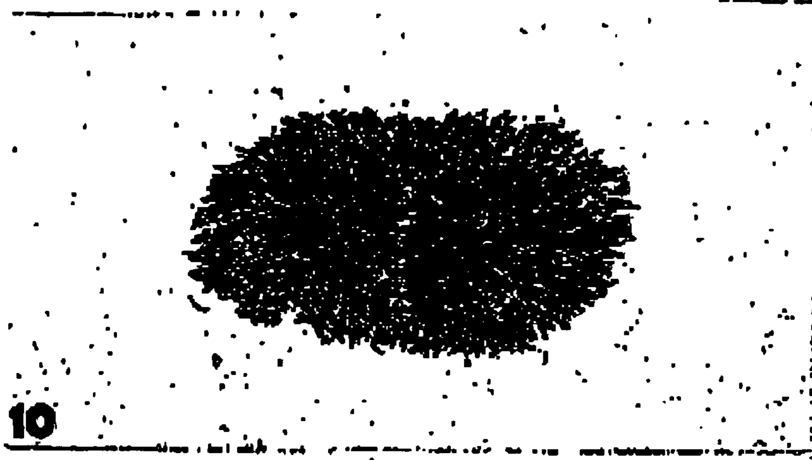
PLATE III



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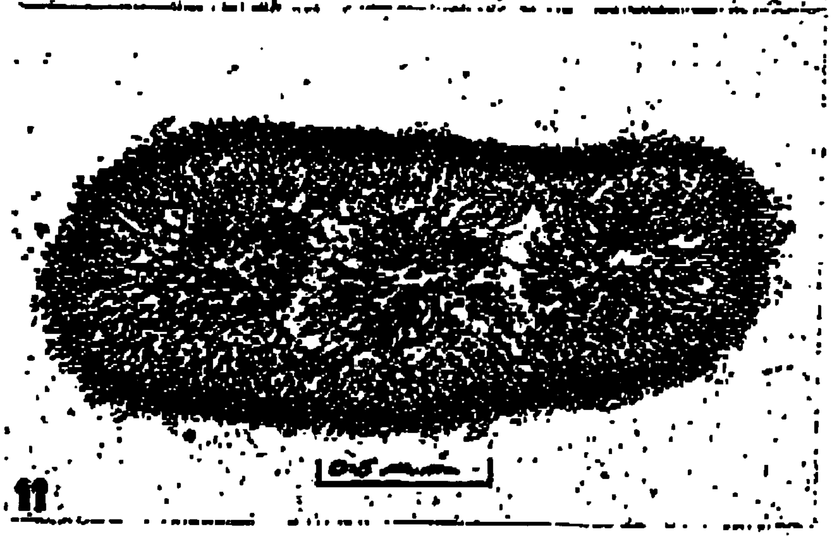

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FIGS. 10, 11. Photomicrographs of cross sections of clumps of pellets. Fermentation age: Fig. 10, 24 hours; Fig. 11, 42 hours.

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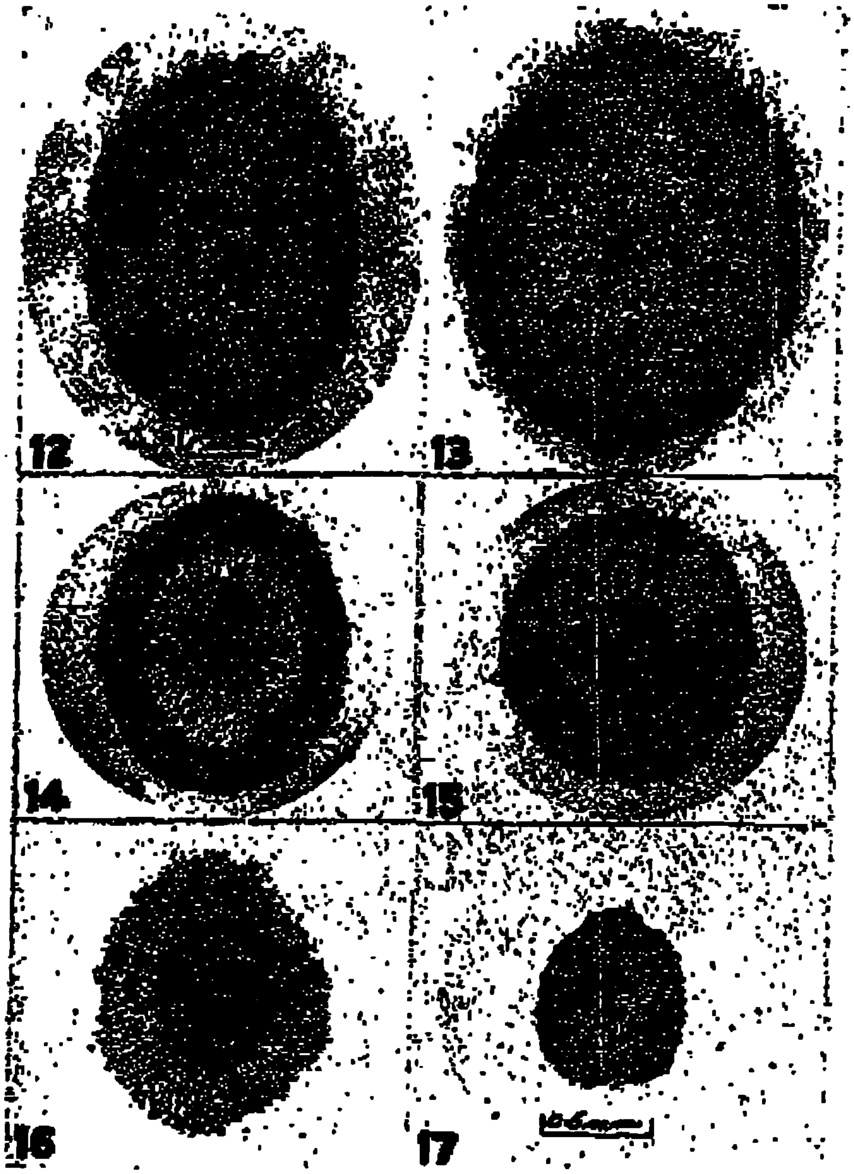
PLATE IV



FIGS. 12, 13. Photomicrographs of cross sections of pellets grown in ferrocyanide-free mash. Fermentation ages: Fig. 12, 72 hours; Fig. 13, 140 hours. Magnification for both figures shown in Fig. 13.

FIGS. 14–17. Photomicrographs of cross sections of pellets grown in mash containing different concentrations of ferrocyanide. Ferrocyanide concentration at start of fermentation: Fig. 14, 15 p.p.m.; Fig. 15, 100 p.p.m.; Fig. 16, 400 p.p.m.; Fig. 17, 600 p.p.m. Fermentation age 72 hours. Magnification for these figures shown in Fig. 17.

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PLATE IV

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PLATE V

FIGS. 18–21. Photomicrographs of cross sections of pellets grown under different oxygen pressures. Oxygen pressures: Figs. 18 and 19, 1.7 atm; Fig. 20, 0.2 atm; Fig. 21, 0.5 atm. Fermentation age—Figs. 18, 20, and 21, 72 hours; Fig. 19, 140 hours.

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(Fig. 7), the dense peripheral growth had reached maximum thickness (0.1–1.5 mm) and the space between it and the now pronounced area of complete autolysis had undergone partial autolysis. An increase in extent of autolysis and in pellet diameter were the only changes occurring with further increases in age (Figs. 8, 9).

The particles of precipitate formed during the ferrocyanide treatment of mash became attached to growing hyphae early in the fermentation and stained densely with crystal violet. The size of the heavily stained area at the center of the pellet in Fig. 5 suggests that the pellets had cleared the mash of precipitate by about the 10th hour of fermentation. As autolysis occurred, the particles moved to existing cells (Figs. 6–9).

Morphological changes in clumped pellets with time were similar to those in separated pellets (Plate III). Until oxygen was turned on, neither autolysis nor dense growth at the surface took place (Fig. 10), whereas shortly afterwards both conditions were evident (Fig. 11). The darkly stained central areas (precipitate particles) of the individual pellets forming the clumps were always roughly spherical (Figs. 10, 11), indicating that clumping occurred at about the time the mash was cleared of precipitate. For the pellet in Fig. 11, the reduction in surface area as a result of clumping was about 20%.

The effect of the ferrocyanide content of the mash on the internal appearance of pellets is shown in Figs. 12–17. The large filamentous pellets that developed in the absence of ferrocyanide (Fig. 3) did not undergo noticeable autolysis at any time and produced dense peripheral growth only after about 100 hours of fermentation (Figs. 12, 13). When the mash contained 15 p.p.m. of ferrocyanide at the start of fermentation (optimum for citric acid production), 3-day-old pellets (Fig. 14) possessed an extensive area of partial and complete autolysis as well as a shell of thick surface growth. In the presence of 100 p.p.m., however, pellets at this age (Fig. 15) were smaller, surface growth was less dense, and the area of autolysis more restricted. The increased inhibition of growth and autolysis obtained with still higher concentrations of ferrocyanide is shown in Figs. 16 and 17.

Oxygen pressure in the fermenter during the acid-producing stage of fermentation had a marked effect on the density of peripheral growth and on rate and extent of autolysis (Plate V). At 1.7 atm oxygen pressure, autolysis of cells at the centers of the pellets was noticeable only after about 6 days of fermentation (Figs. 18, 19), whereas at 1.0 atm pressure the rate of autolysis was much more rapid (Figs. 7 and 9). With an oxygen pressure of 0.5 atm, peripheral growth in 3-day-old pellets (Fig. 21) was less dense than in pellets of the same age developed under higher pressure, but the extent of autolysis was still marked. When the oxygen pressure was reduced further to 0.2 atm, the pellets were larger, possessed a uniform growth density throughout, and did not autolyze (Fig. 20).

Discussion

Formation of dense surface growth and rate and extent of autolysis were the most significant changes that occurred in pellet morphology with changes in fermentation conditions. Autolysis appears to result from the resistance to the passage of nutrients and oxygen to cells inside the pellet by the dense growth

at the surface. Evidence of this is shown in the fact that autolysis did not occur in the absence of heavy peripheral growth (Figs. 12, 13, 16, 17, 20), and in the delay in autolysis that occurred when the oxygen pressure was increased from 1 to 1.7 atm (Figs. 18, 19). The extension of cell life inside the pellet, as a result of an increased oxygen tension, may account partially for the increase in rate of citric acid production previously noted for high oxygen pressures (1). The reason for the formation of concentrated surface growth is not explained by the present results, but such growth was related to ferrocyanide concentration (Figs. 12–15) and to oxygen pressure (Figs. 18, 19), and required for high citric acid yields. This concentrated growth did not occur, and yields of citric acid were low when ferrocyanide was absent and the oxygen pressure less than half an atmosphere.

Acknowledgment

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